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In vitro clonal propagation of *Tanacetum cinerariifolium* and establishment of an *ex situ* collection of selected clones

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Abstract

Dalmatian pyrethrum *Tanacetum cinerariifolium* (Trevir.) Sch. Bip. (Asteraceae) is a perennial herb endemic to the eastern coast of the Adriatic Sea. The species is widely cultivated in many countries for its bioactive compounds pyrethrins, which are used as natural insecticides. Plants derived from seeds vary greatly in pyrethrin content; therefore, the vegetative propagation of high-quality individuals is very important for the establishment of agricultural pyrethrum crops. The present study deals with rapid *in vitro* multiplication of pyrethrum, *ex vitro* adaptation of selected clones and creation of an *ex situ* collection, as a first step towards introducing the species into agriculture in Bulgaria. Seeds from a private *ex situ* collection in Bulgaria and from a natural Croatian population were used as initial material for *in vitro* cultures initiation. Basal MS medium (Murashige and Skoog 1962) or MS supplemented with different concentrations of kinetin and indole-3-butyric acid were used for seed germination and multiplication of one-seed derived clones by consecutive subcultivations. The propagation effectiveness was evaluated as a number of new plants obtained per initial shoot. Considerable losses were noticed due to both endophytic contaminations and necrosis, especially on media supplemented with plant growth regulators. These problems were overcome by medium optimization: adding an antibiotic and modifying the medium to increase the calcium concentration using $CaCO_3$. In the best medium variant (basal MS + 200 mg/L Medaxone + 75 mg/L Ca) no more infected plants were observed, and the percentage of necrotic plants decreased threefold, which resulted in formation of 38.06±10.11 new plants per initial shoot for a period of 7 months. Three hundred and sixty plants were *ex vitro* adapted in a phytotron (88% surviving rate), then 16 plants from 4 selected clones were transferred to the *ex situ* collection and bloomed twice from the very first growing season (June and September). The number of the flower heads increased in the second year of field cultivation and an average of 328±138 capitula per plant were counted for the best clone. The first trials to establish a pilot plantation of pyrethrum are promising.

Keywords
Chrysanthemum cinerariaefolium, *ex vitro* adaptation, *in vitro* micropropagation, nutrient medium modification, plant acclimatization, pyrethrum

Introduction

Dalmatian pyrethrum *Tanacetum cinerariifolium* (Trevir.) Sch. Bip., also known by its synonyms *Pyrethrum cinerariaefolium* Trev., and *Chrysanthemum cinerariaefolium* Bocc (Pignatti 1982; Grdiša et al. 2009; Rahmatullah et al. 2010), is a perennial herb of Asteraceae family. It is endemic to the eastern coast of the Adriatic Sea, distributed from Italy to northern Albania and in the mountainous regions of Croatia, Bosnia and Herzegovina, and Montenegro (Staykov and Ilieva 1961; Nikolić and Rešetnik 2007; Grdiša et al. 2009; Grdiša et al. 2013; Šegota et al. 2016). The species was first introduced into agriculture in Armenia (Casida 1980), and is currently widely cultivated in many countries due to the pyrethrins it contains (Staykov and Ilieva 1961; Fulton et al. 2001a; Grdiša et al. 2009; Casida 2012). The use of pyrethrins as an insecticide is thought to have originated in Persia, where *Pyrethrum roseum* Bieb. and *Pyrethrum garneum* Bieb. were known long ago (Staykov and Ilieva 1961; Wainaina 1995). According to other authors, dried parts of *T. cinerariifolium* were used as insecticide in folk medicine in Croatia (Grdiša et al. 2009).

Natural pyrethrins can be characterized as an excellent means of combating insect pests (Jovetic 1994; Hammond 1996; Palmquist et al. 2012). They are very effective against a wide range of insects (Tattersfield et al. 1929; Arnaudov 1930; Richardson 1931; Greenhill 2007; Cai et al. 2010) acting as a contact insecticide (Grdiša 2009), and the risk of developing resistance is low.

Seed germination of *T. cinerariifolium* is not high, especially in natural populations (Singh and Sharma 1989; Fulton et al. 2001b). In our previous study on seeds gathered from an *ex situ* collection in Bulgaria, it was found that seeds quickly lost their vitality and their germination rate dropped dramatically after several years of storage (Ilinkin et al. 2020). Seeds can also be purchased from pyrethrum breeding programs (British Oxygen Company) with 80% germination rate (Fulton et al. 2001b). However, agricultural crops of *T. cinerariifolium* are usually established by vegetative propagation as plants derived from seeds vary greatly in pyrethrin content (Casida 1973; Ikahu and Ngugi 1988; Lindiro et al. 2013).

Access to high-quality plant material is the main limiting factor for the mass cultivation of *T. cinerariifolium*; therefore the development of an efficient *in vitro* protocol for micropropagation of selected high-yielding plants is a task for many researchers (Lindiro et al. 2013). Most studies have been based on agar-solidified media (Staba et al. 1984; Hussain et al. 1994; Keskitalo 1999) and rarely has liquid media been tested (Staba and Zito 1985; Keskitalo 1999). The characteristics of the culture medium can be a major factor in establishing a highly efficient *in vitro* culture. Some authors (Roest and Bokelmann 1973; Kaul et al. 1990; Hedayat et al. 2009) reported successful propagation of *Chrysanthemum*

species on MS medium (Murashige and Skoog 1962). According to Hedayat et al. (2009) the composition of MS medium is optimal for species belonging to the genus *Tanacetum*. These authors compared the results obtained on three media differing in their basic composition: MS, B5 (Gamborg et al. 1968), and SH (Schenk and Hildebrandt 1972) and noticed the highest weight of fresh biomass on MS medium. The addition of BAP in the medium stimulated the formation of multiple shoots and led to decrease of their length, which confirmed earlier results obtained for *Tanacetum vulgare* L. (Keskitalo 1999).

The aim of the present study was to establish suitable conditions for propagation of *T. cinerariifolium* by *in vitro* methods and to create an *ex situ* collection of selected clones, as a step towards introducing the species into agriculture in Bulgaria.

Materials and methods

Plant material

The initial studies on *in vitro* culture initiation were carried out with seeds of *T. cinerariifolium* taken from a private *ex situ* collection in the village of Bogdan, Bulgaria. Subsequently, seeds from a natural Dalmatian pyrethrum population were used for further experiments (MAPO2821 – Accession number from Croatian Plant Genetic Resources Database), kindly provided by Dr. Martina Grdiša.

Seed sterilization, nutrient media composition and culture room conditions

Seeds were disinfected after standard surface sterilization procedure: 1 min soaked into 70% ethanol, then 10 min into commercial bleach (Cl < 0.5%), and triple rinsed in distilled sterile water for 5, 10, and 15 min.

Seeds gathered from the collection of Bogdan village were germinated on three nutrient media on MS base (Murashige and Skoog 1962): medium MS free of plant growth regulators (PGRs), and two media supplemented with kinetin (Kin, Duchefa, NL) and indole-3-butyric acid (IBA, Duchefa, NL) in different concentrations: 1.0 mg/L Kin and 0.5 mg/L IBA (medium KI), or 0.2 mg/L Kin and 0.1 mg/L IBA (medium K_2I_1).

Seeds originating from the Croatian population were germinated on control MS medium (MS), three MS-based media supplemented with the antibiotic Medaxone (active compound ceftriaxone sodium) in concentrations 100 mg/L (medium MS100M), 200 mg/L (medium MS200M), and 300 mg/L (medium MS300M), and medium B5 (Gamborg et al. 1968) free of PGRs.

In consequence, medium MS200M supplemented with three concentrations of Ca (30, 75, and 120 mg/L) added as $CaCO_3$ were tested during subcultivations along with medium MS200M.

All media contained 30 g/L sucrose and were solidified with 6.5 g/L Plant agar (Duchefa, NL), they were autoclaved at 121 °C, under 1 atm, for 20 min, and then put into plastic containers with passive ventilation. Four sets of 100 seeds were used for each medium variant.

In addition, cultivation in temporary immersion system (TIS) was tested with shoots taken from *in vitro* culture with Croatia origin growing on agar-solidified MS medium MS200M. Six containers RITA were used, 10 shoots per container, with 200 ml liquid medium MS200M, flooding the shoots for 5 minutes 4 times a day.

Conditions in the culture room were: 16/8 h light/dark regime and temperature of 23±2 °C around the clock. Clones obtained by *in vitro* shoot multiplication were selected on the base of the number of surviving plants.

Culture subcultivation

Two months after the start of the experiments seedlings were cut to upper and lower parts and roots were removed, thus obtaining explants from stem segments. One-seed derived clones were obtained by several consecutive subcultivations on fresh medium with the same composition as the corresponding initial medium, in plastic containers: newly formed shoots were separated, and the longer ones were additionally cut to segments. Explants developed into new plantlets. Propagation coefficient (PC) was calculated as an average of *in vitro* plantlets obtained per explant. Subcultivations were performed at intervals of about two months for seedlings rising from the seeds gathered from Bogdan collection, and at intervals of three weeks for those originating from the Croatian population. Subcultivations on MS-modified media containing higher concentrations of Ca were done every month. Infected and necrotic plants were removed periodically during each subcultivation.

Ex vitro adaptation and outdoor acclimation

Three hundred and sixty plants belonging to four selected clones were obtained from seeds originating from the Croatian population germinated on medium MS200M and multiplied on medium MS200M supplemented with 75 mg/L Ca, were potted in soil mixture (Light mix Biobiss, France) and *ex vitro* adapted first in a growth chamber (POL-EKO Aparatura, Poland) for 6 weeks (under strict temperature and light control, and gradual decrease of the air humidity from 90% to 60%) and then in a room phytotron. Surviving plants were transferred to an unheated greenhouse and in October 2019 four plants per clone were acclimated outdoors in the *ex situ* collection of IBER, planted at a distance of 40 cm from each other and 40 cm between the rows. The numbers of both stem ramifications and flower heads per individual were assessed during the first and the second flowering in 2020. The numbers of the flower heads were compared between the clones and between the years 2020 and 2021.

Statistical analyses

Microsoft Excel (ver. 16.6) was used to calculate the regression equations, and ANOVA test (Microsoft Excel) to demonstrate statistical significance ($p < 0.01$) in the regressions shown. LSD Post Hoc (SPSS, version 26) test was used to verify statistically significant differences in seed germination ($p < 0.05$).

Results

In vitro cultivation on media supplemented with PGRs (initial seeds from Bogdan village)

Nineteen one-seed-derived *in vitro* clones of pyrethrum were multiplied on media MS, KI, and K_2I_1, up to eight subcultivations (Fig. 1). Explants formed one or more plantlets, and the presence of PGRs in the medium stimulated shoots formation and elongation; however, endophytic bacteria were noticed in some cultures, which caused necrosis and loss of entire clones. Obviously, the presence of Kin and IBA enhanced the microorganisms' multiplication as well, as 81.3% of the plantlets obtained on medium K_2I_1 and 65.0% of those obtained on medium KI died due to microbial contamination (Fig. 2), although losses were also high on the control MS medium. Propagation coefficients were calculated up to the third and the sixth subcultivations for media K_2I_1 and KI, respectively, because there were no more surviving plants on these media. It is noteworthy that with each subcultivation the number of plants dropping out of the experiment due to infections or necrosis increased. Both necrotic shoots and necrotic rooted *in vitro* plants were observed. The highest percentage of surviving plants was noticed on PGR-free MS medium, but after the eighth subcultivation, all plantlets were affected by necrosis or microbial contamination.

In vitro cultivation on media supplemented with antibiotic (initial seeds from Croatia)

The germinating rates of the seeds on the five tested media: basal MS or B5, and MS media supplemented with different concentrations of Medaxone, were similar (Fig. 3). The effect of the antibiotic was immediate, as at the time of the first subcultivation the number of the infected seedlings on medium MS100M was lower than on the control

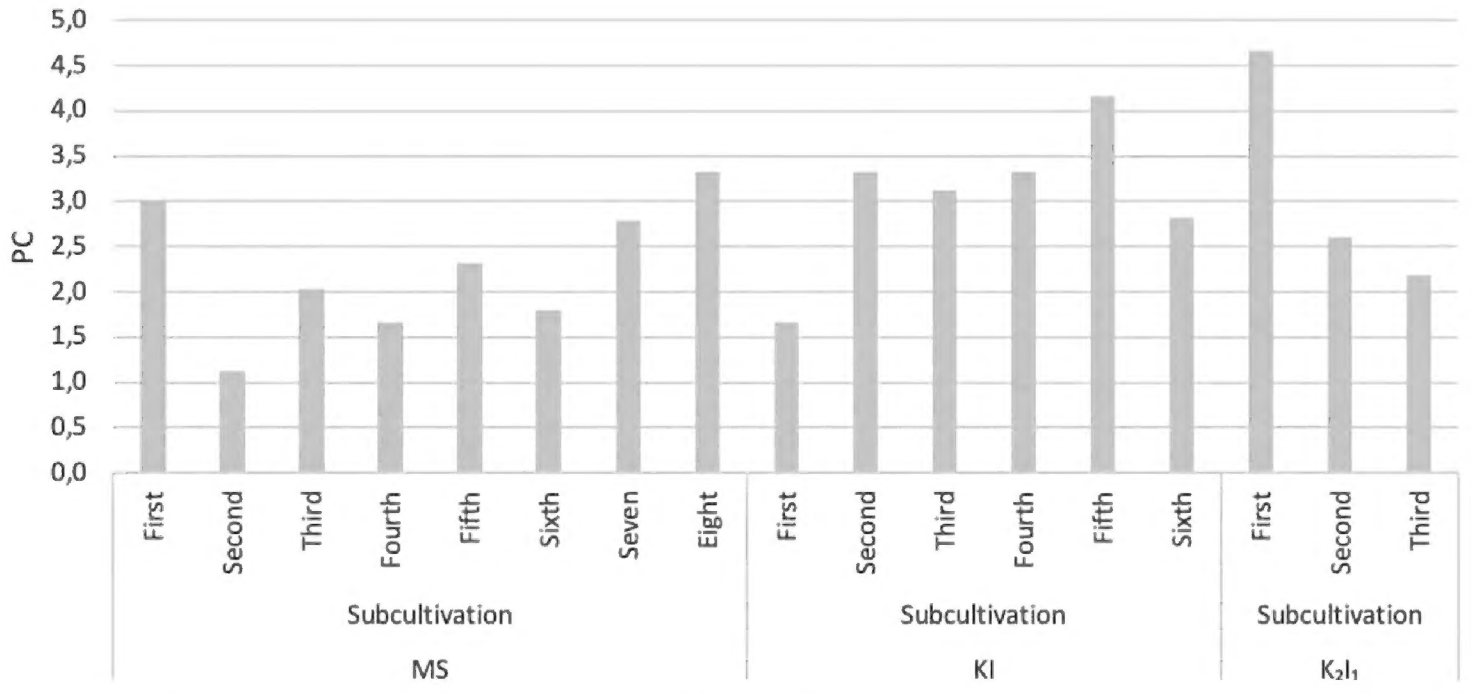

Figure 1. Effect of the plant growth regulators Kin and IBA on shoot multiplication rate.

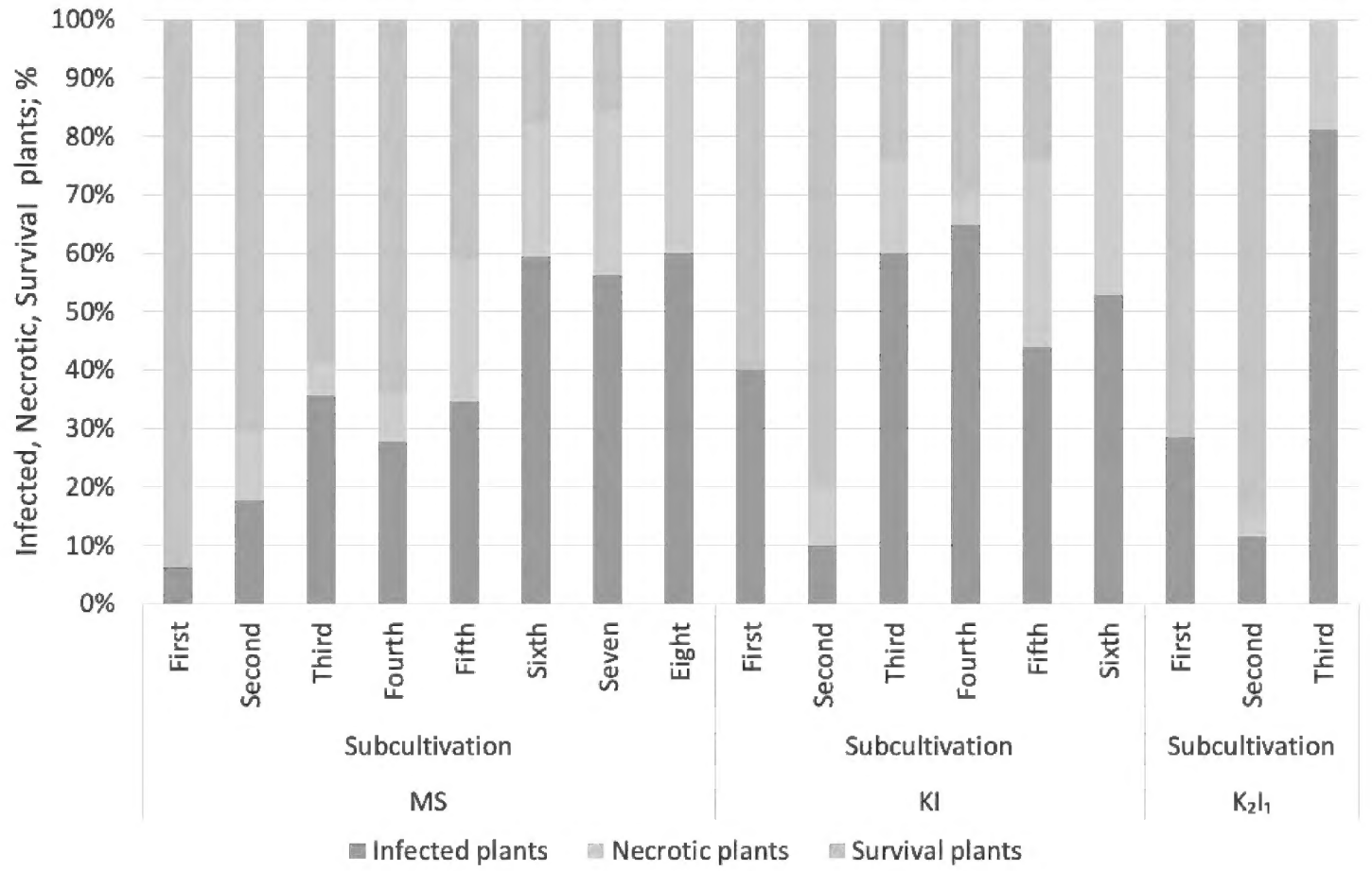

Figure 2. Effect of the PGRs Kin and IBA on plantlets survival in long-term *in vitro* culture.

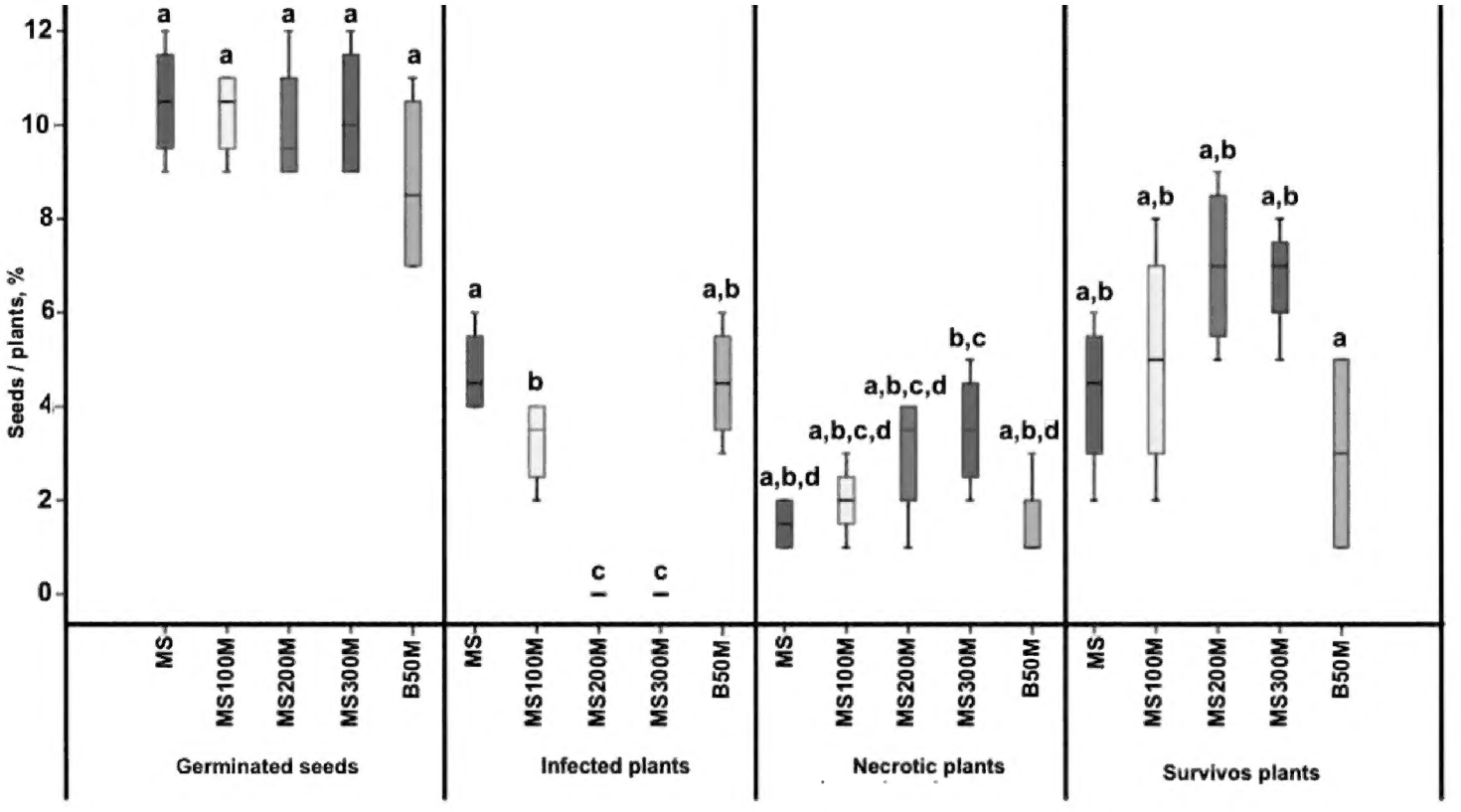

Figure 3. Seed germination and seedling survival evaluated as percentages from all tested seeds ($p < 0.05$).

MS medium ($p < 0.05$), while no infected seedlings were observed on the two media containing higher Medaxone concentrations (Fig. 3). The highest percentages of the surviving *in vitro* seedlings were on the same two media: MS200M and MS300M. However, with increasing antibiotic concentration, there was a clear trend towards an

increase in the percentage of necrotic plants, therefore medium MS200M was assessed as the best one for culture initiation, ensuring both bacteria elimination and the highest number of surviving seedlings.

Medium composition slightly influenced the time needed for seed germination, but there was no distinct peak of germination energy in any of the variants (Fig. 4) as the maximal percentage of germinating seeds in one day was 3%. Seeds began to germinate earliest on MS control medium (on day 12.5±1.7) but the duration of seeds' germination was the longest on this medium (15.5±2.5 days). Seed germination on all media containing antibiotic began about the 15th day and its duration was shorter (12.5±2.9 days for MS100M, 11.5±2.5 days for MS200M, and 11.5±0.6 days for MS300M). The longest time it took for seeds to start germinating was on B5 medium: 16.8±2.2, and the period of seed germination was 14.0±2.2 days.

In vitro subcultivation began with different numbers of seedlings on the tested media (between 12 and 28) due to the differences in their survival rates. Each seedling was separately multiplied during 8 consecutive subcultivations, which resulted in many one-seed-derived clones (Fig. 5A, B). The average propagation coefficients of all subcultivations are presented for each medium on Fig. 6. Cultures grown on antibiotic-supplemented media showed a tendency toward a slight decrease in the multiplication rates compared to that of the control culture on basal MS medium. Among the cultures grown on the media without antibiotic, the one on medium B5 had a lower PC, which was similar to that of the culture grown on MS100M containing Medaxone at the lowest concentration. The coefficient of variation of multiplication rate was higher in the antibiotic-free cases (8.82% for medium MS and 8.83% for medium B5) than that estimated for the media containing Medaxone (4.75% for MS100M, 6.08 for MS200M, and 6.22% for MS300M).

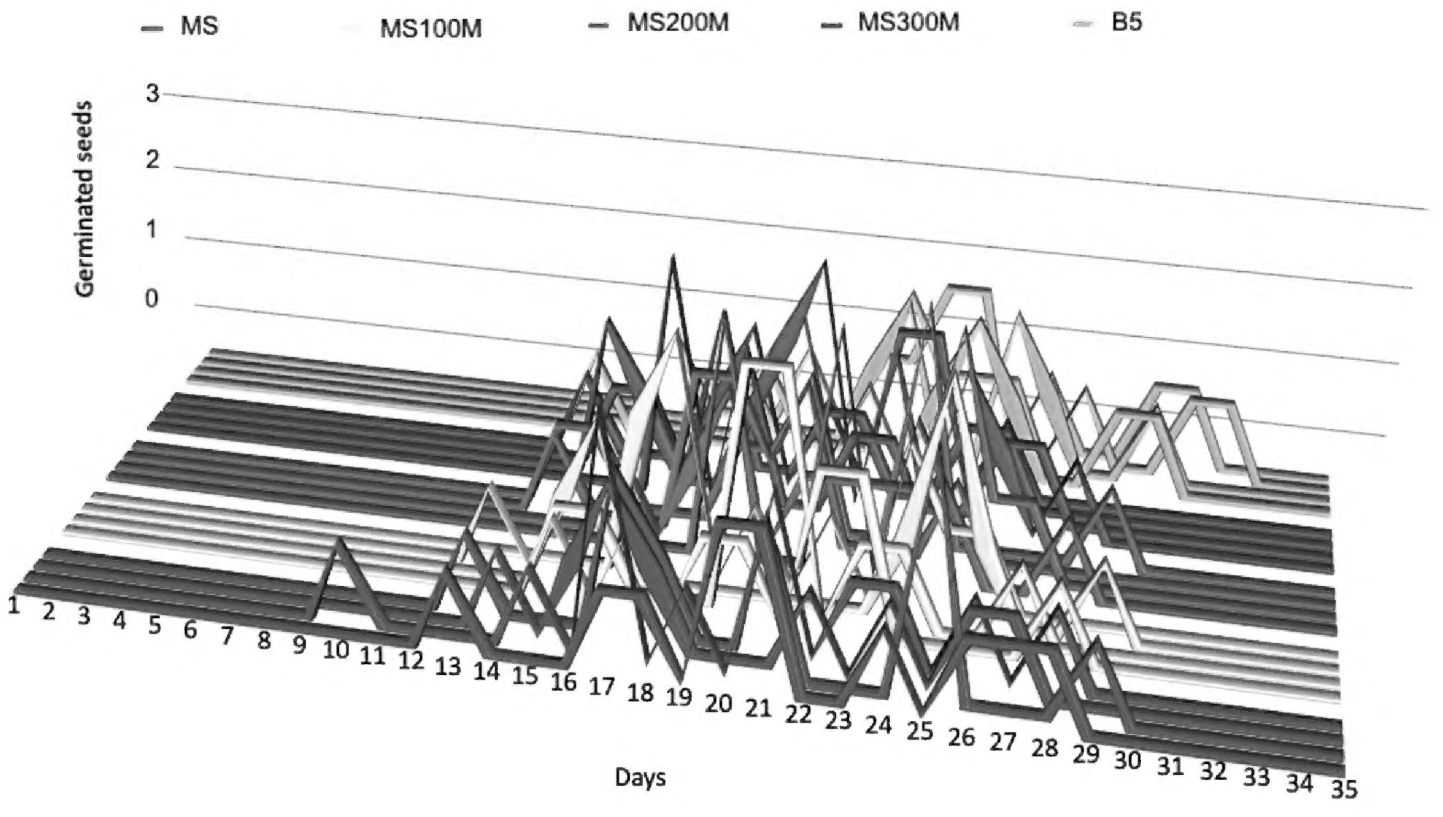

Figure 4. Effect of nutrient media composition on germination energy.

Figure 5. *In vitro* propagation of *T. cinerariifolium* plants (origin: Croatian natural population) **A** *in vitro* culture on agar-solidified medium **B** multiplication of *in vitro* clones **C** cultivation in TIS **D** *ex vitro* adaptation in a growth chamber **E** adaptation in a room phytotron **F** acclimated plants in the *ex situ* collection, first flowering in June 2020 **G** flowering in June 2021.

At every subcultivation, there were dropping out shoots. On MS and B5 antibiotic-free media only a few plantlets survived due to both bacterial infections and necrosis, the importance of which varied from one subcultivation to another (Fig. 7). At the last subcultivation, the number of surviving plantlets on MS and B5 media were 15 and 3, respectively, while on media supplemented with Medaxone their numbers were significantly higher: 58 on medium MS100M, 671 on MS200M, and 114 on MS300M, all of them well-shaped, rooted, and ready to be *ex vitro* adapted. Bacterial infection was completely eliminated in media containing 200 or 300 mg/L Medaxone; however, the highest antibiotic concentration seemed to inhibit shoot multiplication, as the number of *in vitro* plants at the 8th subcultivation was almost 6 times higher on the medium supplemented with 200 mg/L Medaxone. Medium MS200M was chosen for further optimization, as the number of necrotic plantlets remained high.

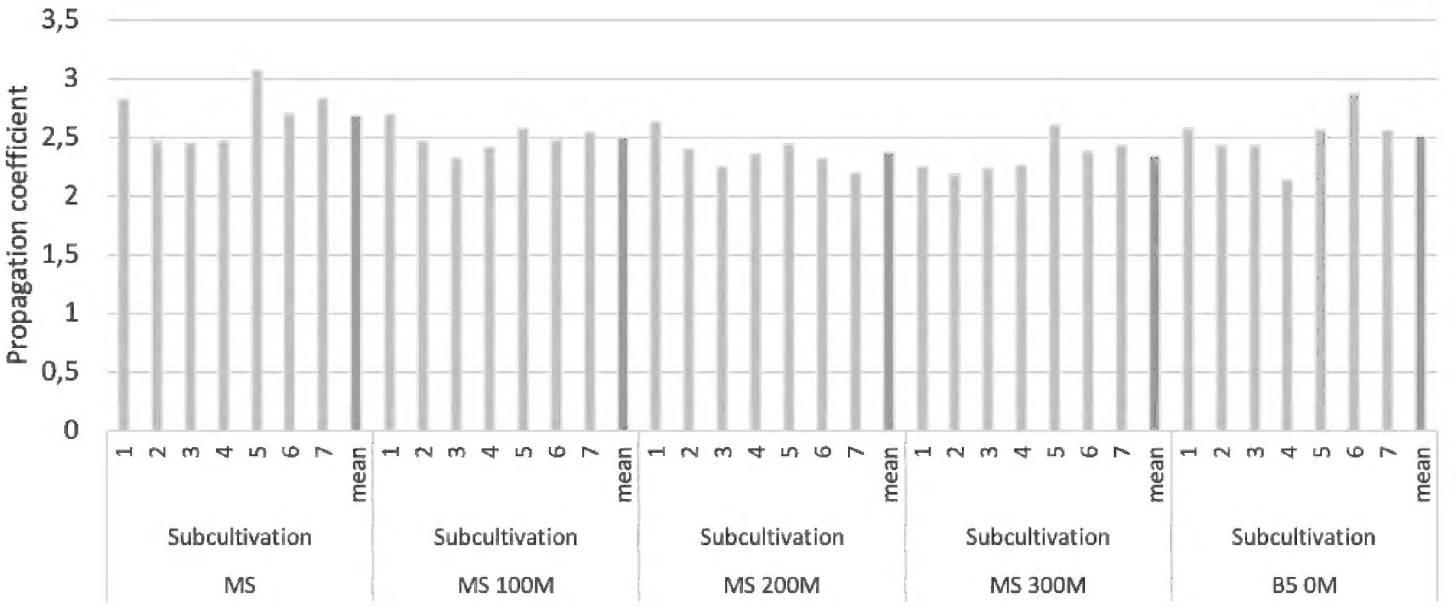

Figure 6. Effect of antibiotic concentration on the propagation rate of pyrethrum *in vitro* cultures.

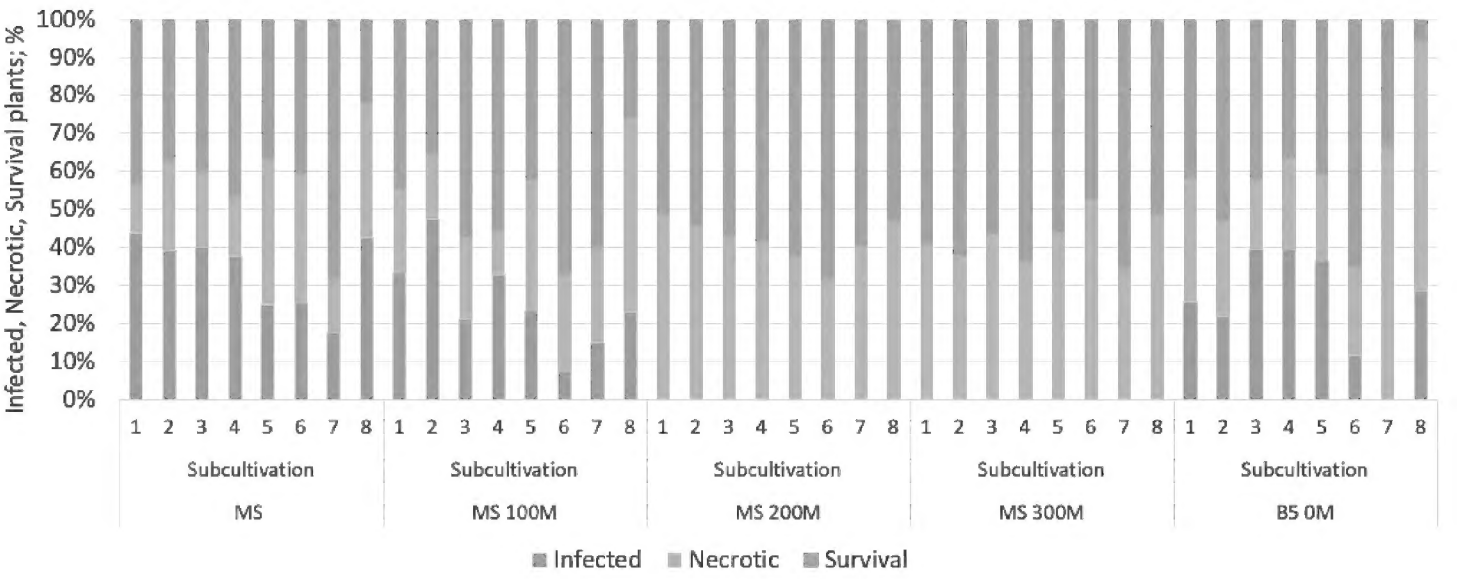

Figure 7. Effect of antibiotic concentration on survival of pyrethrum plants under long-term *in vitro* culture conditions.

Pyrethrum *in vitro* cultivation in TIS, RITA containers with liquid medium MS200M, did not improve the propagation coefficient of the clones tested. Plantlets formed long roots and grew faster, but the percentage of necrotic plants remained high (Fig. 5C). Agar-solidified medium turned out to be more suitable.

In vitro cultivation on MS modified medium, effect of Ca

The MS medium was modified by increasing the concentration of Ca, added as $CaCO_3$, and further optimization of medium composition was performed on MS supplemented with 200 mg/L Medaxone. The propagation coefficients of the four pyrethrum *in vitro* clones multiplied on the three media supplemented with different concentrations of Ca and on medium MS200M, remained similar, between 1.9 and 2.1 (Fig. 8).

However, the number of necrotic plants was significantly influenced (Fig. 9). The variation of PC decreased with the increase in the concentration of Ca supplemented: on medium MS200M with no added Ca it was 6.04; while at 30 mg/L Ca it was 5.40; at 75 mg/L Ca – 4.80, and at 120 mg/L Ca – 4.24. The lowest percentage of necrotic plantlets was noticed on medium containing 75 mg/L Ca, followed by that on medium with 120 mg/L Ca. However, with the increase of subcultivations, the percentage of necrotic plants on Ca-supplemented media increased, and in the case of the two higher concentrations these trends were statistically significant. At the highest concentration (120 mg/L Ca), this correlation was much more pronounced (Fig. 9), therefore the modified MS medium supplemented with 75 mg/L Ca and containing 200 mg/L Medaxone was selected as the best one for *T. cinerariifolium in vitro* multiplication. No more infected plants were observed, and the percentage of necrotic plants decreased threefold, which resulted in the formation of 38.06±10.11 new plants per initial shoot for a period of 7 months.

Ex vitro adaptation and outdoor acclimation

In vitro multiplied plantlets rooted spontaneously on all media tested (Fig. 5A). Several thousands *in vitro* plants were obtained in one year on the optimized medium. After 6 weeks of *ex vitro* adaptation in the growth chamber, 88% of the plants strengthened and reached about 10 cm in height (Fig. 5D). Almost all plants survived during the next steps of adaptation in the room phytotron and the greenhouse (Fig. 5E). Sixteen plants from 4 clones were transferred outdoors to the *ex situ* collection of IBER, where they grew and bloomed in June at the first season (Fig. 5F). Secondary flowering was also observed in autumn but with a smaller number of

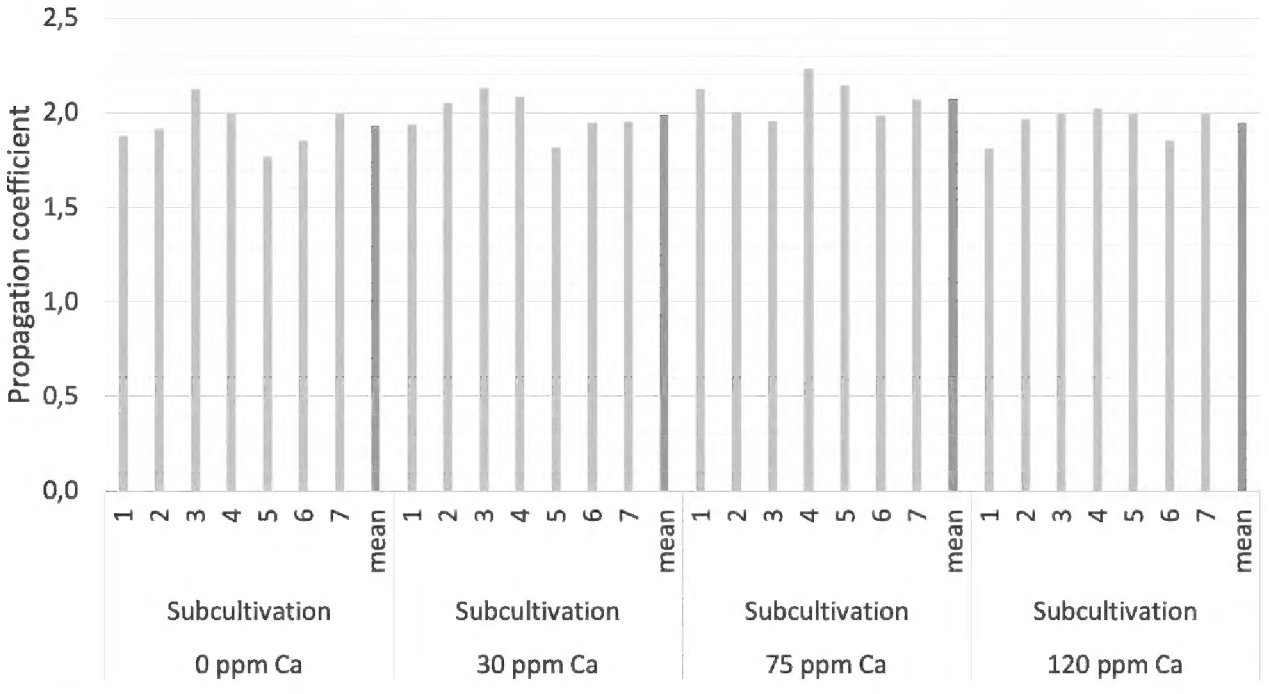

Figure 8. Effect of increasing calcium concentration in the medium on pyrethrum multiplication capacity *in vitro*.

capitula (Fig. 10). Differences in stem branching and in the number of capitula were observed not only between clones, but also between individuals within the same clone (Fig. 11). The uneven number of capitula of the plants from one and the same clone was most probably due to the faster growth of some individuals at the very beginning, which overshadowed the neighboring individuals. Plants of Clone 4 suffered from the shadow of a tree nearby, which led to delay of their growth and finally to their death. Plants of Clone 1 flowered slightly before those of the other clones (Fig. 12). Plants' growth continued and an average of 328±138 flower heads per individual plant formed in the second year for the best clone (Clone 3), which was 6 times more compared to the first year (Fig. 5G). Plants in the greenhouse also bloomed, but their growth was limited by the size of the pots. Some plants did not survive the next winter outdoors and were replaced by plants growing in the greenhouse. The following spring and summer were quite rainy, which had a bad effect on their development.

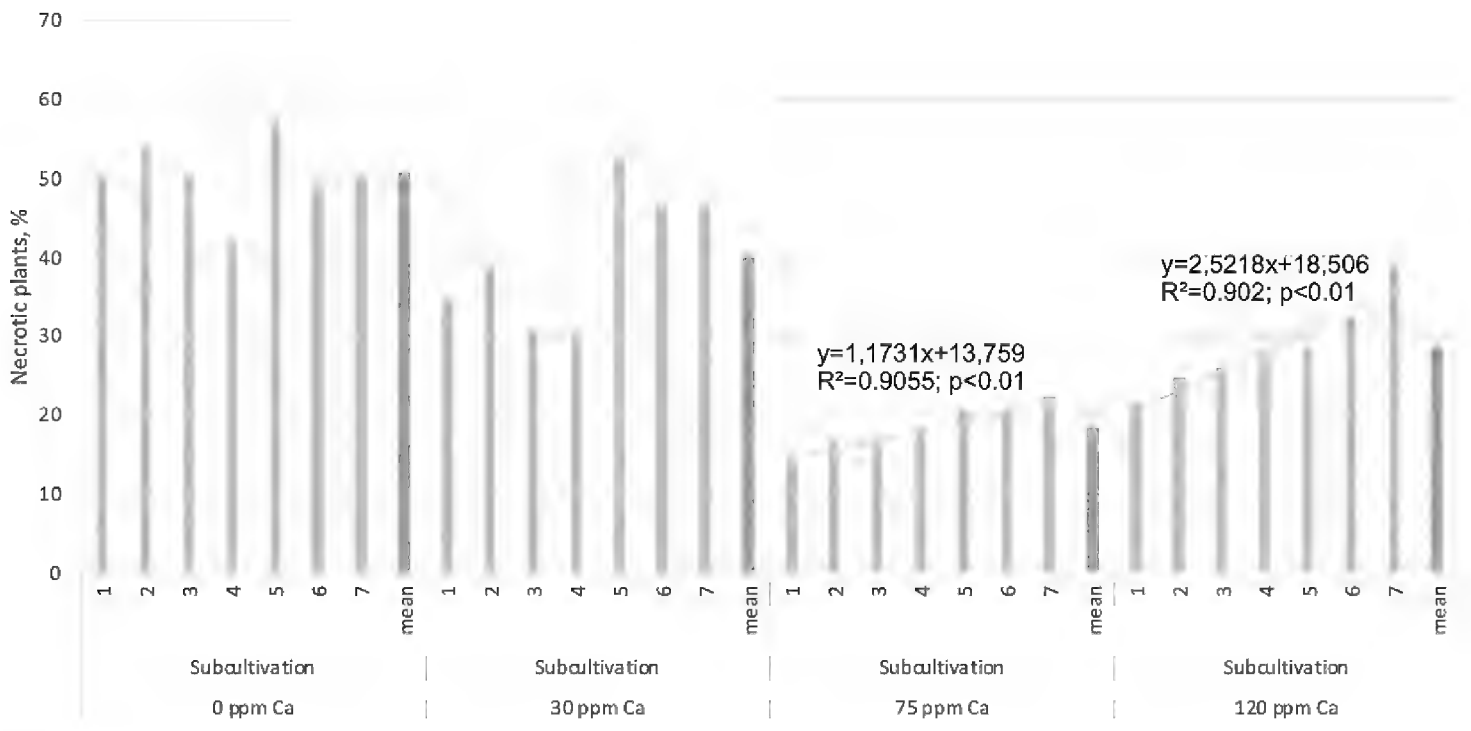

Figure 9. Effect of increasing calcium concentration in the medium on plant necrosis frequency ($p < 0.01$).

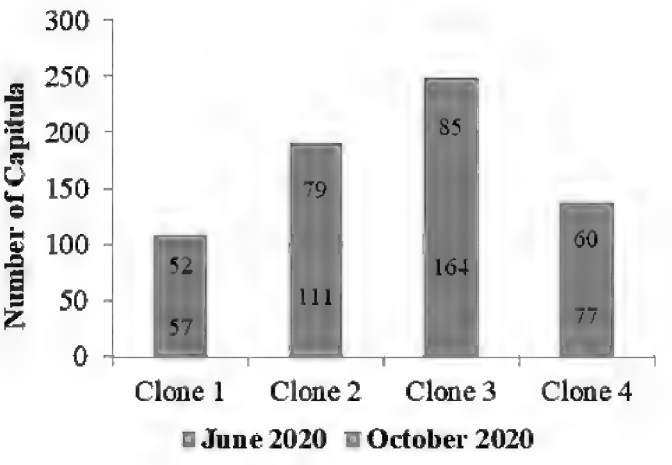

Figure 10. Primary and secondary flowering in the first year of cultivation in the *ex situ* collection.

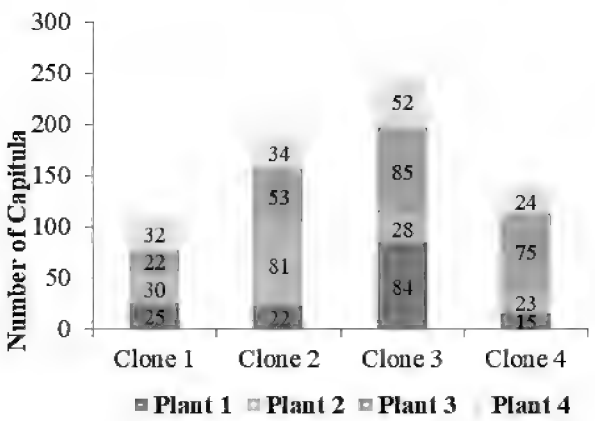

Figure 11. Total number of capitula in the first year of cultivation in the *ex situ* collection.

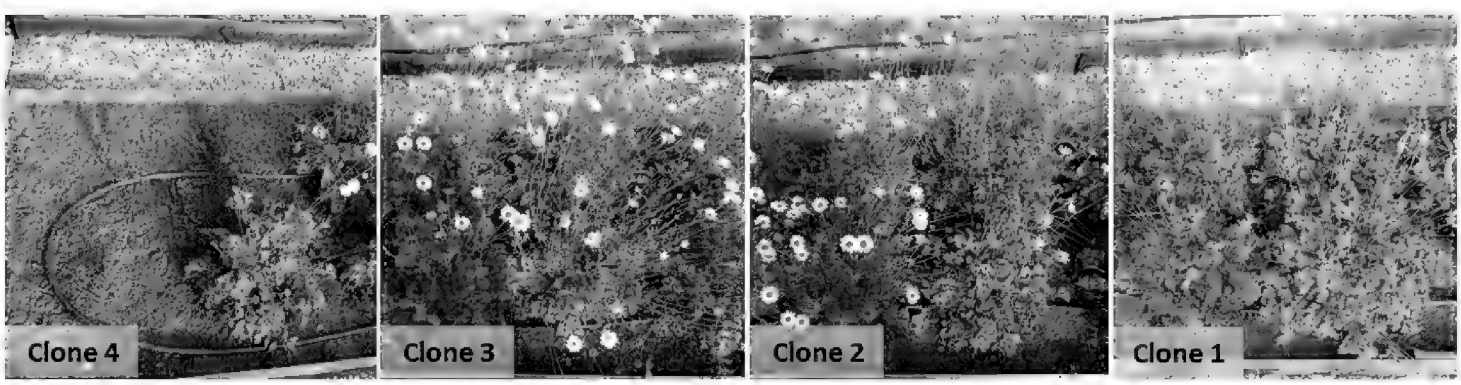

Figure 12. Clones during their secondary flowering in autumn 2020.

Discussion

For their normal growth and development, plants need macro salts and microelements, the optimal concentrations of which may depend on the species. Among media for *in vitro* plant propagation, MS and B5, as well as their modifications, with or without addition of PGRs, are most commonly used. Bacterial contamination could be critical for *in vitro* culture initiation despite disinfection of primary explants (Hennerty et al. 1987; Misaghi and Donndelinger 1990; Keskitalo 1999). Usually, the composition of media for plant cultures is not optimal for microorganisms or suppresses their development, so their presence in the culture can go unnoticed for a long time (Leifert and Waites 1992; Leifert et al. 1994; Isenegger et al. 2003). In general, bacterial infections appear soon after the cultures are transferred to fresh media, because during subcultivations the roots are removed and the plants are cut into segments, making them weaker, as in the case of our experiments. Antibiotics are habitually added to the medium to control bacterial contamination, but they can alter the morphogenesis of *in vitro* cultures and slow down their growth (Teng and Nicholson 1997; Eady and Lister 1998; Keskitalo et al. 1998; Teng and Teng 2000; Bergant et al. 2005). The effectiveness of the antibiotics varies depending on the plant species, and sometimes even on the species genotype (Keskitalo et al. 1998; Bergant et al. 2005).

In our opinion, necrosis observed *in vitro* in pyrethrum cultures was due to an insufficient amount of calcium in the most commonly used media. *T. cinerariifolium* is not pretentious regarding soil conditions (with the exception of waterlogged soils), but it develops remarkably well on calcareous soils (Hennigsberg 1941; Astadzhov et al. 1980). According to some authors, the natural populations of the species are on

carbonate soils and karst terrains (Sladonja et al. 2014). The physiological functions of calcium are related to cell division, normal functioning of cell membranes and activation of enzymatic reactions that positively affect photosynthesis. Calcification strengthens plant cell walls, thereby increasing plant resistance to infections (Gorbanov et al. 2005; George et. al. 2007). In addition, calcium manifests synergism with NO_3, i.e. facilitates its uptake and hinders the absorption of some elements such as K^+, Na^+, Mg^{2+}, Fe^{2+} and Zn^{2+}. Calcium deficiency in the presence of potassium and magnesium can lead to significant changes in the normal functioning and growth of roots and root hairs (Gorbanov et al. 2005). Another negative effect of this insufficiency can be the severe limitation of the shoot tips growth and even their death due to hyperhydricity, which can be avoided by frequent subcultivation (George et. al. 2007). According to some authors (Sha et al. 1985; Singha et al. 1990), the degree of changes may differ among different genotypes of the same species. On the other hand, excessively high carbonate content in soils and high pH of the soil solution can negatively affect plant development and production of pyrethrum flower heads (Sastry et al. 1988). In our experiments, the optimal result was obtained at the intermediate calcium concentration of the three variants tested. It is important to note that calcium is also essential for *in vitro* morphogenesis, especially when using PGRs such as auxins and cytokinins (George et al. 2007). This may explain the faster death of plants subcultured on media containing PGRs, without antibiotic and supplemental calcium. In *in vitro* cultures, the addition of calcium as $CaCl_2$ is not preferable, as it may even lead to a significant increase in chlorine content to levels of chlorine toxicity (George et al. 2007). On the other hand, addition of calcium as $CaCO_3$ and subsequent lowering of pH, which usually occurs in *in vitro* cultivation, could also lead to an increase in chlorine content. This may be a likely explanation for the presence of credible trends (at concentrations of 75 and 120 mg/L Ca) for an increase in necrotic plants with increasing cultivation time.

Some authors recommended for *in vitro* shoot rooting MS medium free of PGRs or B5 supplemented with 2 mg/L NAA (Rostiana and Seswita 2007; Hedayat et al. 2009). Information on *ex vitro* adaptation and outdoor acclimatization of regenerated pyrethrum plants is scarce. Survival of about 2/3 of the plants was reported, with the remark that the process can be greatly improved using a phytotron with tightly controlled environmental parameters (Catalano et al. 2011). Our results proved the positive effect of the strict control of the ambient conditions in the growth chamber during the first weeks of *ex vitro* adaptation, when *in vitro* plants are most vulnerable to changes in air humidity and temperature. Soil characteristics are of importance for seed germination and plant development. A pot experiment involving several different soil types proved that Rendzic Leptosol was the most suitable for pyrethrum seed germination (Ilinkin 2019). The soil mixture used for *ex vitro* adaptation in pots (Light mix Biobiss, France) was suitable for pyrethrum plants and they survived several years in the unheated greenhouse and flowered. The field plot conditions were less appropriate as the soil was loamy-sandy, poor in carbonates. Plants in the *ex situ* collection bloomed twice a year; however during the third growing season some of them remained small. Sunlight proved to be crucial for the survival and vigorous growth of *T. cinerariifolium* plants. Plants of

this species should be planted at a greater distance from each other to avoid their death. Wandahwa et al. (1996) also reported the importance of the edaphic conditions for successful cultivation of the species and capitula yield. These authors propagated selected clones vegetatively in soil for 3–4 months, and then seedlings were planted in a permanent place of cultivation at 60 cm inter-row spacing and 30 cm between plants in the row. Up to 10% of the plants died and were replaced with new seedlings to maintain the plantation. Planting on ridges was recommended by Kroll (1963) to provide better soil aeration and avoid waterlogging. In our case, the dead of some plants was probably due to the rainy spring and summer and the shadow of the neighboring tree.

Data on the influence of nitrogen, phosphorus and potassium fertilization reported for different pyrethrum growing countries are conflicting. Clone-specific responses to nitrogen and phosphorus fertilization were also noted (Ngugi and Ikahu 1989). Our results related to some features of the clones we selected, such as the number of capitula, are consistent with the observations of these authors and confirm the assumption that the study should be conducted at individual level.

Conclusion

A protocol for *in vitro* micropropagation of *Tanacetum cinerariifolium* has been established, starting with seeds, and several one-seed-derived clones have been obtained by multiple consecutive subcultivations. The optimization of the nutrient medium composition was of crucial importance for the successful *in vitro* cultivation. Shoot loss due to both endophytic bacteria and necrosis was overcome by adding an antibiotic and modifying the calcium concentration in the medium, according to the specific requirements of pyrethrum. The first attempts to establish a pilot plantation of pyrethrum are promising, as *ex vitro* adaptation of the plants was easy and the outdoor acclimated plants bloomed twice from the very first growing season. However, field cultivation conditions need to be improved, as pyrethrum plants are shade intolerant and direct sunlight is crucial for their survival. The number of flower heads increased during the second year of cultivation in the *ex situ* collection, and some differences were found between the clones tested. Flower heads were sampled from each individual for analysis of pyrethrin content. More field experiments are needed to select highly productive individuals in terms of both number of flower heads and concentration of pyrethrins. Selected individuals should be further *in vitro* propagated to produce seedlings for pyrethrum plantation.

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